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Human macrophages studies

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An abbreviated version of this protocol was published in Science in Oct 2020

Mammalian lipid droplets are innate immune hubs integrating cell metabolism and host defense

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Detailed protocol

Macrophage infection assay:

On the day prior to infection:

Preparation of the macrophages

1. Harvest human monocyte-derived macrophages (HMDMs) by removing media and lifting cells with 10 ml PBS and a syringe with a blunt 18g needle. Note: depending on the donor, HMDM can be easy or difficult to lift.
2. Wash HMDM twice with PBS and resuspend in penicillin/streptomycin (p/s)-free Iscove's Modified DMEM (already containing Glutamine), 10% feta bovine serum (FBS) and 150 ng/ml recombinant human colony stimulating factor-1 (CSF-1) (IMDM media) before the cell count.
3. Plate out HMDM in 24 well TC coated plates at desired cell concentration (e.g. 200,000 – 300,000 cells/well) in a total volume of 900 µl p/s-free IMDM media.
4. Place the cells in an incubator (37 ºC, 5% CO2) overnight.

Preparation of the bacteria

1. Under aseptic conditions: add 10 ml of LB broth to 2 x 50 ml falcon tubes.
2. Pick a single colony of bacteria and add it to one of the 50 ml tubes, the other will be a sterile control.
3. Place both falcon tubes in a bacterial shaking incubator overnight (37 ºC).

On the day of infection:

1. Take 1 ml of the overnight culture, add 9 ml PBS under aseptic conditions, spin for 10 min at 2740G, remove supernatant, wash bacteria again in another 10 ml PBS, re-suspend pellet in 10 ml p/s-free IMDM media and determine A_{600}.
2. Characterisation (blank with IMDM media):

Salmonella Typhimurium $A_{600} = 1$ AU $= 8.5 \times 10^8$ cfu/ml

Escherichia coli (K12) $A_{600} = 0.6$ AU $= 1 \times 10^9$ cfu/ml

(E. coli need a reculture with 1:1 volume of fresh LB broth for 1-2 h before infection or there will be too many dead bacteria in the culture)

1. Dilute bacteria to a known/workable concentration in media.
2. Add appropriate # bacteria in a 100 µl volume to each well (e.g. if plating 200,000 HMDM, MOI 100 would be $2x10^7$ bacteria in 100 µl).
3. Incubate at 37 ºC for 1 h.
4. Meanwhile, determine the multiplicity of infection (MOI) – see below.
5. After 1 h, aspirate media from wells, wash twice with 1 ml high dose gentamicin (200 µg/ml)-containing IMDM medium.
6. Add another 1 ml high dose gentamicin (200 µg/ml)-containing IMDM medium, incubate at 37ºC for 1 h.
7. Aspirate media from wells, wash wells twice with 1 ml p/s-free IMDM.
8. Add 1 ml low dose gentamicin (20 µg/ml)-containing IMDM medium to each well. Incubate at 37ºC for the appropriate time (depending on your end time point).

At appropriate time points:

1. Aspirate media and wash gently twice with 1 ml PBS.
2. Add 1 ml PBS/0.01% Triton X100 to each well.
3. Leave for 5 min in the incubator.
4. Pipette up and down to homogenise the lysed cells. Aliquot lifted cells into wells of a 96 well plate in triplicate (~200 µl/well).
5. Perform dilutions on 96 well plate and plate out 5 µl drops of sample onto LB agar plates in triplicate.
6. Incubate plate at 37 ºC overnight.

NB: you should plate out at least 2-3 dilutions/sample i.e neat and 1/10 or 1/100 etc. The dilution depends on how high your MOI is.

Result:

1. Count colonies.

Assessing MOI:

1. Add 180 µl PBS to 8 wells (non-TC 96 well plate).
2. Add 20 µl of inoculum used in the experiment to infect cells to well 1 and serially dilute by a factor 10 (20 µl in 180 µl) to well 8.
3. Plate out 5 µl (in triplicate) from wells 6 and 7.
4. Incubate at 37 ºC overnight.
5. From here you can work out your actual MOI: bacteria # (cfu/ml) = # colonies x dilution factor x 200 (volume multiplier to 1 ml, from the 5 µl that was plated).

Materials Required:

Penicillin/streptomycin (p/s)-free Iscove's Modified DMEM (containing Glutamine) (ThermoFisher Catalogue# 12440061), 10% FCS and 150 ng/ml CSF-1 (IMDM media).
Gentamicin (Life Technologies Cat#15710072)
PBS
Triton X100
Class 2 biosafety cabinet
37˚C, 5% $C0_2$ tissue culture incubator
24 well TC-treated plates
96 well plate
Falcon tubes
Spectrophotometer
Cuvettes
Centrifuge with swing-out rotor
LB broth
LB agar plates

Notes:

1. The concentration of CSF-1 required will vary, depending on the source.
2. Each condition is performed in either duplicate or triplicate (and from each well, technical replicates are performed to count cfu).
3. MOI determination is essential to confirm actual MOI used and because high MOI can kill macrophages.
4. Make sure p/s-free media is used throughout the experiment.
5. For stationary phase bacteria, use overnight (O/N) cultures. For log phase bacteria, inoculate 1 mL of an O/N culture into 10 mL of broth and culture at 37°C for approximately 4 h.
6. The absorbance to bacterial number ratio above is for *Salmonella* and *E. coli* only. Use different values for other bacteria.
7. When infection assays are being performed on different cell populations that are being directly compared, make sure the different cell populations are also plated out for an MTT assay (to control for cell numbers).
8. Ensure separate 24 well plates are used for different time points.
9. In initial experiments, monitoring cell death [e.g. by a lactate dehydrogenase (LDH) release assay] is useful to make sure that cellular manipulations do not cause excessive cell death. When performing LDH assays, make sure that samples from the 1 h and 2 h washes are collected and assessed, otherwise rapid cell death during this time will be missed (25 ul of the supernatant is sufficient).

CD14 human monocyte isolation:

Setting up for the procedure:

1. Book tissue culture (TC) hood – it can take ~8 h to isolate monocytes after receiving blood (depending on the number of donors and the blood composition of the donor).
2. Make-up MACS buffer (PBS containing 2 mM EDTA and 0.5% fetal bovine serum, FBS) and cool to 4°C.

Performing the procedure:

A. Ficoll density centrifugation to isolate peripheral blood mononuclear cells (PBMC).

1. Add 12.5 ml of Ficoll-Paque into 50 mL Falcon tubes (4 tubes needed per buffy coat).
2. *Dilute the buffy-coat.* Divide ~60 mL buffy coat into 3 x 50 mL Falcon tubes (~20 mL per tube). Top up with saline to 40 mL (dilution ½) and mix well, either by inverting or pipetting up and down during the next step.
3. Using a 25 mL pipette, gently overlay 30 ml of the diluted blood onto the Ficoll (set expiration at lowest speed, angling tubes almost horizontally and applying blood to the side wall of tubes). This is a **CRITICAL** step. The Ficoll and the blood should NOT mix (if they do, you won't be able to separate the PBMC by centrifugation).

4. Centrifuge for 45 min at 200G (room temperature), **Brake set to SLOW (EXTREMELY IMPORTANT)**.
5. After this centrifugation step, you should be left with a) a substantial pellet of red blood cells (Ficoll makes them clot so they are dense enough to pellet); b) if the blood was fresh, you may see a white layer of neutrophils on top of the RBC below the Ficoll; c) a Ficoll layer; d) a fluffy white layer that contains the required PBMC (monocytes and lymphocytes); and e) a layer of supernatant: dilute plasma, which also contains platelets. Aspirate ~10 mL of the plasma from each tube using a 10 mL pipette until 0.5 cm from the PBMC ring (don't let the tip go below the 20 mL mark).
6. Collect the monolayer into 2 x 50 mL tubes (per donor) using a sterile transfer pipette or 25 mL pipette. Collect as much as possible without collecting too much Ficoll.
7. Top each tube up to 50 mL with saline, **mix well by invertingthe tube** and centrifuge for 10 min at 600G (10 °C), with the **brake on**.
8. Using a 25 mL pipette, carefully discard the supernatant (the post-Ficoll pellet will be very loose). Using saline, combine the two tubes into one, top up to 50 mL with saline then centrifuge for 10 min at 500G to 600G (10°C), with the**brake on**. If there has been an issue with the donor and there is a large amount of RBC, you may lyse RBC at this point (however, this will likely result in significant cell death).

B. MACS separation. **Positive Selection for CD14+ monocytes.**

All solutions should be pre-cooled. MACS buffer: PBS + 0.5% FBS, 2 mM EDTA.

1. Centrifuge cells at 400G for 10 min, completely remove supernatant (using 25 mL and 1000 µL pipettes).
2. Resuspend pellet in 10 mL MACs Buffer.
3. Add 300 µL Human CD14+ microbeads.
4. Mix and incubate for 60 min at 4°C (use a rocker in a cold room to keep cells in suspension).
5. Top up the falcon tube to 50 mL with MACS buffer, centrifuge for 5 min at 400G (4°C).
6. Remove supernatant with a 25 mL pipette, resuspend pellet in 2 mL MACS buffer.
7. Pass the cell suspension through a cell strainer, adding extra MACs buffer if necessary to wash through.
8. Pre-load each LS column with 3 mL MACS buffer (2-3 columns per donor).
9. Divide the cell suspension across the LS columns and run through, wash with 9 mL (3x washes of 3 ml) MACS buffer.
10. Elute positively-selected cells in 5 mL MACS buffer by removing column from magnetic field and applying plunger force.
11. Count purified monocytes.
12. Pellet monocytes, resuspend in complete IMDM and plate for differentiation or experiments.

C. FACs staining to check purity (Optional).

1. Take an aliquot for FACS staining to check purity. The monocyte yield is usually ~10-15% PBMC, with >95% being CD14+.

D. Monocyte differentiation into macrophages:

1. Plate monocytes at ~15 million cells per 10 cm TC dish in 15 mL complete IMDM + CSF-1 (or GM-CSF if required).
2. Monitor cells and top-up with 5 mL complete medium containing CSF-1 to make a total of 20 mL at day 4.
3. Culture for a total of 6 days. At day 6, there are usually two distinct cell morphologies visible: flat, spread, adherent macrophage-like cells, and round, loosely adherent monocyte-like cells. The balance between these two varies depending on the blood donor. If you take the loosely adherent cells and plate them overnight on fresh plates with CSF-1, they generally take on a striking macrophage morphology. For this reason, all cells are considered as monocyte-derived macrophages, although they may have slightly different characteristics. Harvest on day 6 (in culture medium to maximise yield through retaining both adherent and non-adherent cells; or in 10 mL PBS to recover adherent cells only) and replate for experiments on day 7.
4. HMDM yield from CD14+ monocytes: anywhere from 30-80%. This primarily seems to depend on how fresh the blood was to begin with.
5. Generally harvest and use HMDM from days 6-7.

Materials required:
Blood (~60 mL buffy coat from Australian Red Cross Blood Service)
Saline
Ficoll Paque Plus (NB light sensitive) (Merck Catalogue# GE17-1440-03)
Class 2 biosafety cabinet
37 ˚C, 5% $C0_2$ tissue culture incubator
Complete IMDM Media *(Iscove's Modified DMEM containing Glutamine), 10% FCS, 1% Pen/Strep)* (ThermoFisher Catalogue# 12440061)
Centrifuge with swing-out rotor and capacity to remove brake
QuadroMACs separator LS (Miltenyi Biotech Catalogue# 130-091-051) and Multi Stand
MACS buffer – ice-cold (Sterile Saline or PBS with 0.5% FCS and 2 mM EDTA)
LS MACS columns (Miltenyi Biotech Catalogue# 130-042-401)
CD14+ MACS beads (Miltenyi Biotech Catalogue# 130-050-201)
Cell strainer 40 microns (BD Falcon Catalogue# 352340)
Falcon tubes
TC dishes

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1. Bosch, M. , Parton, R. G., Pol, A. and Sweet, M. (2021). Human macrophages studies. Bio-protocol Preprint. bio-protocol.org/prep1261.
2. Bosch, M., Sánchez-Álvarez, M., Fajardo, A., Kapetanovic, R., Steiner, B., Dutra, F., Moreira, L., López, J. A., Campo, R., Marí, M., Morales-Paytuví, F., Tort, O., Gubern, A., Templin, R. M., Curson, J. E. B., Martel, N., Català, C., Lozano, F., Tebar, F., Enrich, C., Vázquez, J., Pozo, M. A. D., Sweet, M. J., Bozza, P. T., Gross, S. P., Parton, R. G. and Pol, A.(2020). Mammalian lipid droplets are innate immune hubs integrating cell metabolism and host defense . Science 370(6514). DOI: 10.1126/science.aay8085